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Patentanmeldung Nr. Patent application No. Demande de brevet n°

03005186.6

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
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Si aucun titre n'est indiqué se referer à la description.)

Anaplastic lymphoma kinase assay, reagents and compositions thereof

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ANAPLASTIC LYMPHOMA KINASE ASSAY, REAGENTS AND COMPOSITIONS THEREOF

The present invention provides an assay for measuring the kinase activity of the Anaplastic lymphoma kinase (ALK). More specifically the invention is directed to methods, reagents and compositions for detecting the tyrosine-phosphorylating activity of ALK. The assay is particularly useful for 5 in vitro screening and identification of potential ALK inhibitors.

BACKGROUND OF THE INVENTION

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase, believed to play an important role in the development and function of the nervous system. ALK is normally expressed in the central nervous system, 10 with peak expression during the neonatal period. However, due to chromosomal translocations, ALK is also aberrantly expressed and activated in some cancers in the form of oncogenic fusion proteins. ALK fusion proteins are responsible for approximately 5 - 10% of all non-Hodgkin's lymphomas. The annual incidence of ALK positive lymphomas is about 100 000 15 worldwide, with 2000 - 3000 new cases occurring in EU countries. ALK is an excellent candidate for therapeutic intervention, as it plays an essential role in oncogenicity and its normal expression is mostly restricted to the central nervous system. Hence, a specific ALK inhibitor could be an efficient treatment for ALK positive lymphomas with few associated clinical side 20 effects. For the identification of potential ALK inhibitors it is highly desirable to develop a suitable assay for assessing directly the inhibitory effect of compounds on enzyme activity.

DESCRIPTION OF THE INVENTION

According to the invention, an *in vitro* kinase assay specific for ALK 25 and useful for screening compounds that modulate ALK activity is provided.

This assay is based on the use of a peptide substrate which is readily phosphorylated by ALK and on the subsequent detection of the phosphorylated product thus obtained. In a preferred embodiment of the invention the assay is an ELISA whercin the phosphorylated product is
5 detected by immunochemical reactions.

In order to generate a selective ALK substrate, peptides reproducing the sequence of ALK activation loop (aa 1274-1294: ALK_HUMAN, Q9UM73, swiss-PROT) were synthesized and tested. The peptides

ARDIYRASFFRKGGCMLPVK (SEQ ID N. 1) and

10 ARDIYRASYYRKGGCMLPVK (SEQ ID N.2) were particularly effective as ALK substrates showing a phosphorylation degree higher than that of polyGlu/Tyr, a random polymer which is known to be a good substrate for most tyrosine kinases. The first object of the invention is therefore a peptide having an amino acid sequence selected from SEQ ID N. 1 and SEQ ID N. 2.

15 A further object of the invention is a method for detecting ALK tyrosine kinase activity, which essentially comprises the steps of:

i) incubating the ALK protein or a functional derivative thereof with a peptide selected from SEQ ID N. 1 and 2 in conditions suitable for phosphorylation of the peptide;

20 ii) detecting the phosphorylated peptide thus formed.

As used herein, "ALK functional derivative" means any modified form of ALK protein, for example a truncated or conjugated form or a fragment thereof, which maintains the catalytic activity of unmodified ALK. The functional derivative should preferably contain the entire catalytic domain of
25 ALK spanning residues 1116-1392 of ALK sequence (Q9UM73). The portion of ALK protein stretching from residue Leu¹⁰⁷³ to Ala¹⁴⁵⁹ is preferably used. When produced by recombinant gene technology using the baculovirus-based expression system, this ALK fragment shows a correct folding (confirmed by

CD spectra) and an effective catalytic activity.

In addition, a preparation containing a constitutively active form of ALK may be used instead of the purified protein or its functional derivative. Such a preparation is preferably a cell lysate, which could be used to a) assess 5 for ALK activity in whole cells expressing ALK or ALK fusion proteins and b) assess for the effect of potential inhibitors on ALK within a cell, by treating ALK-expressing cells with different compounds and then assaying cell lysate for ALK kinase activity.

To detect the phosphorylated peptide in step ii), the phosphorylation 10 reaction of step i) can be performed in the presence of a radioactive reagent, such as [$\gamma^{32}\text{P}$]ATP or [$\gamma^{33}\text{P}$]ATP, whereby a radioactive product is formed which is easily detected by radiometric techniques. Alternatively, an immunoreaction can be carried out, which comprises the formation of an 15 immune complex between the phosphorylated peptide and an anti-phosphotyrosine antibody. This anti-phosphotyrosine antibody can be radioactively labelled or conjugated to a reporter enzyme, such as horse-radish peroxidase, beta-galactosidase, alkaline phosphatase or glucose oxidase, thereby allowing the direct detection of the antibody and thus of the 20 phosphorylated peptide by measuring the specific radioactivity or enzymatic activity. As a further alternative, the anti-phosphotyrosine antibody may be detected indirectly by a second antibody recognizing the anti-phosphotyrosine antibody and carrying a radioactive label or a reporter enzyme, or by an enzyme conjugated streptavidin which recognises a biotin label on the anti-phosphotyrosine antibody.

25 In a preferred embodiment the invention provides a method for detecting ALK tyrosine kinase activity which comprises the steps of:

- a) adhering a peptide of SEQ ID N:1 or 2 to a solid phase;
- b) incubating the solid phase with an ALK fragment extending from

Leu¹⁰⁷³ to Ala¹⁴⁵⁹ in suitable conditions for tyrosine phosphorylation;

- c) washing the solid phase;
- d) incubating the solid phase with an anti-phosphotyrosine antibody (primary antibody) in conditions suitable for antigen-antibody binding;
- e) washing the solid phase;
- f) incubating the solid phase with an enzyme-conjugated antibody (secondary antibody) recognizing the anti-phosphotyrosine primary antibody in conditions suitable for binding of primary antibody to secondary antibody, so that a ternary immune complex is formed;
- g) washing the solid phase;
- h) measuring the enzymatic activity of the immune complex wherein the measured activity is proportional to the amount of tyrosine phosphorylation.

This assay, which is properly an ELISA-based kinase assay, utilizes enzyme-antibody conjugates. The conjugated enzyme cleaves a substrate to generate a colored reaction product that can be detected spectrophotometrically by measuring the absorbance of the colored solution, which is proportional to the amount of phosphotyrosines.

Solid phases or supports which can be used according to the invention comprise plastic material (reaction plates, wells, vials), polystyrene, lattice and magnetic beads, colloidal metal particles, glass and/or silica surfaces and others.

The ELISA-based ALK assay is preferably used for the screening of compounds that modulate the tyrosine-phosphorylating activity of ALK, in particular for screening ALK inhibitors.

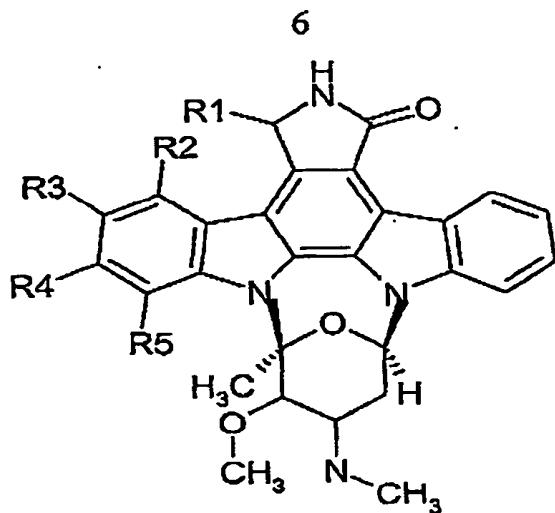
In a preferred embodiment the invention is therefore directed to a

method for the identification of compounds that modulate ALK tyrosine-kinase activity, wherein the ALK assay described above is carried out in the presence of a candidate compound or of a compound known to stimulate or inhibit ALK tyrosine kinase activity (control). Specifically, the method for identifying compounds that modulate ALK tyrosine-kinase activity comprises the steps of

- 5 i) incubating ALK protein or a functional derivative thereof with a peptide selected from SEQ ID N. 1 or 2, preferably SEQ ID N. 1, in the presence of a candidate compound, in conditions suitable for phosphorylation of the peptide;
- 10 ii) quantifying the phosphorylated peptide thus formed;

Compounds that inhibit ALK activity are selected as potential therapeutic agents for use in the treatment of ALK-related tumors, such as anaplastic large cell lymphomas and non-Hodgkin lymphomas. The ALK-modulating activity 15 of a candidate compound can be compared to that of a reference compound (control), which is assayed under the same conditions as the candidate compound. Staurosporine (Meggio F. et al, Eyr. J. Biochem. (1995) 234, 317-322), which proved effective as an ALK inhibitor at 0.2 – 0.3 μ M concentration, can be used as a positive control when screening other 20 compounds for ALK inhibitory activity.

Studies of molecular modeling gave important indications as to the pattern of substitutions required to improve staurosporine affinity and specificity towards ALK protein. The most effective structures are represented in the following general formula (I):



Wherein R1 and R2, independently of one another, are selected from halogen, preferably chlorine, phenyl or C1-C3 alkyl optionally substituted with one or more halogens; R3 is hydroxyl; R4 is hydroxyl or hydroxymethyl; R5 is C1-C3 alkyl, optionally halo-substituted, or benzyl.

5 The compounds of formula (I) antagonize the binding of ATP to the tyrosine kinase domain of ALK within oncogenic fusion proteins such as NPM-ALK, ATIC-ALK, CTLC-ALK, TFG-ALK or other sporadic fusions with tropomyosins. Accordingly, they can be used in the preparation of a medicament for the treatment of ALK-related tumors, especially anaplastic 10 large cell lymphomas and non-Hodgkin lymphomas.

In a different embodiment of the invention, compounds (I) are used in an ALK-assay for the screening of ALK-inhibitors, as herein disclosed. High-throughput screenings can also be implemented using the ALK assay of the invention.

15 According to a further aspect, the invention is directed to a kit for carrying out the ALK assay described above. The kit consists of a packaged combination of reagents in predetermined ratios. Typically the kit will contain a peptide of SEQ ID N: 1 or 2, optionally immobilized on a solid phase, the anti-phosphotyrosine antibody and, if necessary, an antibody labelled with an 20 enzymatic or radioactive label. In addition the kit may contain reagents for

colorimetric or radiometric reactions, buffers, controls such as staurosporine or a derivative thereof, diluents, detergents, stabilizers and any other component useful for setting up and carrying out the assay. The relative amounts of the various reagents may be varied to optimize the sensitivity of the assay. Particularly the reagents may be provided in solid or liquid preparations, such as solution, suspension, dispersion, dry powders, lyophilized preparation. The kit components are supplied in single or separate containers. The kit may also include instructions for carrying out the assay.

DESCRIPTION OF THE FIGURES

10 **Figure 1:** purification and activity of recombinant His-tagged rALK
A) Silver stained 10% SDS-PAGE gel showing marker (M), dialysed and pooled DEAE column fractions loaded onto the HiTrap column (load), HiTrap column fractions (numbers indicate fraction number). B) Radioactive autophosphorylation assay of purified rALK. Lane 1: autoradiograph of 32P-labelled rALK. Lane 2: silver staining of the same sample as in Lane 1.

15 **Figure 2:** kinetics for ALK kinase with peptides

Figure 3: Detection of rALK activity using the ELISA based kinase assay

20 A) Phosphorylation of peptide SEQ ID N.2 by purified rALK. An ELISA kinase reaction was performed with or without 0.5 μ g purified rALK, 15 μ g peptide SEQ. ID N.2 or no peptide, at 30°C for 30 mins. The graph shows absorbance at 450 nm. B) The kinetics of ALK peptide substrate phosphorylation. An ELISA kinase reaction was performed using 206 μ M peptide SEQ. ID N.2 or 42 μ M peptide SEQ. ID N.1 with 0.1 μ g of purified rALK. The reaction was stopped by adding EDTA after 0, 0.5, 2, 5, 10 and 15 mins.

25 **Figure 4:** The effect of peptide substrate concentration on level of phosphorylation

The ELISA assay was performed in the presence of 0.2 µg purified rALK and 0 - 105 µM peptide SEQ ID N.1 (A) or 0 - 315 µM peptide SEQ ID N.2 (B), at 30°C for 15 mins.

Figure 5: The effect of rALK concentration on substrate phosphorylation

An ELISA assay was performed using 206 µM peptide SEQ ID N.2 or 42 µM peptide SEQ ID N.1 and rALK ranging from 0 - 225 ng, at 30°C for 15 mins.

Figure 6: Inhibition of rALK activity by Staurosporine

An ELISA assay was performed using 42 µM SEQ ID N.1, 20 ng rALK, at 30°C for 15 mins in the presence of 0 - 5 µM staurosporine or an equivalent volume of solvent, DMSO. Graph shows A450 normalised to the untreated control.

EXPERIMENTAL RESULTS

Peptide synthesis

Peptides were synthesised by automatic solid phase synthesis utilising 9-fluorenlmethoxycarbonyl (Fmoc) chemistry with an Applied Biosystems Model 431A synthesiser on 4-hydroxymethyl-copolystyrene-1% divinylbenzene-resin (0.95 mmol/g, 0.05 mmol).

Fmoc amino acids (0.5 mmol) were activated by 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (1 eq) and 1-hydroxybenzotriazole (HOBr) (1 eq) in the presence of DIPEA (2 eq).

Peptidyl-resins (100 mg) were cleaved and deprotected in a mixture containing 5 ml of trifluoroacetic acid, 0.375 gr of phenol, 0.125 µl of 1,2-ethanedithiol, 0.250 µl of thioanisole, and 0.25 µl of water for 2 hours. The reaction mixture was filtered onto a tube containing ethyl ether cooled at 0°C. Precipitated peptides were separated by centrifugation and washed with fresh ether.

Crude peptides (50-100 mg in 10 ml of water) were pumped onto a preparative RP-column (prepNova-Pak HR C18, 6 μ m, 25x10 mm, Waters) and eluted with a linear gradient of 10% to 45% acetonitrile at 12 ml/min.

The purity of peptides was > 90% as judged by analytical RP-HPLC on 5 a 5 μ m, C18 Symmetry300 column, 4.6x250 mm (Waters) using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid at 1 ml/min. Peptide molecular weights were confirmed by mass spectroscopy using a matrix-assisted laser-desorption ionisation time-of-flight (MALDI-ToF) spectrometer (Maldi-1; Kratos-Schimadzu, Manchester, UK).

10 **Production and purification of recombinant ALK**

A portion of ALK, stretching from amino acids Leu¹⁰⁷³ to Ala¹⁴⁵⁹, has been cloned into the baculovirus transfer vector, pBlueBacHis2C (Invitrogen). Using this vector and the MaxBac® 2.0 baculovirus expression system (Invitrogen) we have produced recombinant baculovirus and expressed 15 recombinant ALK (rALK) protein in Sf9 (*Spodoptera frugiperda*) insect cells. This protein has a theoretical molecular weight of 49 kDa and contains the predicted catalytic domain of ALK (Ile¹¹¹⁶ to Val¹³⁹²) fused to a 6-histidine-tag (His-tag). The autophosphorylation activity of His-tagged rALK, which is indicative of correct folding, has been verified by anti-phosphotyrosine 20 immunoblotting of whole cell lysates and an *in vitro* radioactive kinase assay.

To produce protein for purification, Sf9 cells were infected with recombinant virus at a multiplicity of infection (MOI) of 5. Cultures were incubated for 3 days at 27°C and then harvested by centrifuging at 400 g for 10 minutes at 4°C. Cell pellets were stored at -80°C until use. To lyse the 25 cells, cell pellets were resuspended in hypotonic buffer (Buffer A) containing 50 mM Tris-HCl pH 8, 20 mM NaCl and protease inhibitors (Pepstatin, Benzamidine, Leupeptin and Aprotinin). After 30 minutes incubation on ice, the cell suspension was centrifuged at 1500 g for 15 minutes at 4°C and the

supernatant was filtered through a 0.45 μ m filter. The filtrate was then loaded onto an 80 ml anion exchange Fast Flow DEAE-sepharose (Sigma) column in the presence of Buffer A at a flow of 1.5 ml/min using the AKTA FPLC system (Amersham-Pharmacia Biotech). Bound proteins were eluted 5 in gradient of NaCl ranging from 20 to 200 mM. Fractions were analysed for the presence of ALK protein by immunoblotting. Positive fractions were pooled and dialysed for 3 h at 4°C in 1 litre of native binding buffer (20 mM sodium phosphate pH 7.8, 500 mM NaCl and PI), changing the buffer every hour. Dialysed fractions were then loaded onto a HiTrapTM-nickel affinity 10 chromatography column (Amersham-Pharmacia Biotech), previously equilibrated with native binding buffer supplemented with 50 mM imidazole and 20 mM β -mercaptoethanol. A flow of 0.5 - 1 ml/min was applied to the column. After washing the column with native binding buffer bound proteins were eluted using a linear gradient of imidazole ranging from 50 to 200 mM. 15 Fractions were then analysed for the presence and purity of rALK by SDS-PAGE and silver staining. Figure 1A shows an example purification of rALK.

Finally, positive fractions were dialysed in 50 mM Tris pH 7.4, 100 mM NaCl, 10% glycerol, 20 mM β -mercaptoethanol and protease inhibitors, and 20 stored at -80°C until use. To verify that purified rALK was active we have performed a radioactive autophosphorylation assay. A reaction mix containing 25 μ l of a rALK positive fraction, 6 μ M cold ATP, 1.2 mM DTT, 10 μ Ci [γ -³²P]ATP, 25 mM Hepes pH 7.5, 10 mM MgCl₂ and 10 mM MnCl₂ was incubated at 30°C for 30 minutes. The reaction was stopped by adding 25 Laemmli buffer and heating at 95°C for 5 minutes. The samples were resolved on a 10% SDS-PAGE gel and transferred to an ImmobilonTM-P membrane. Radioactively labelled proteins were visualized by autoradiography as shown in Figure 1B.

Peptide phosphorylation (radioactive assay)

Peptides and the random polymer polyGlu/Tyr (1:4) were phosphorylated in 30 μ l of a medium containing 50 mM Tris/HCl, pH 7.5, 5 mM MnCl₂, 10 μ M Na-vanadate, 30 μ M [γ ³²P]ATP or [γ ³³P]ATP (specific activity 1000 cpm/pmol) and 10 units of rALK [one unit was defined as the amount of enzyme transferring 1 pmol phosphate per min to polyGluTyr (0.1 mg/ml)]. The reactions were terminated after 10 min of incubation at 30°C by spotting 25 μ l of the mixture onto P81 phosphocellulose papers, which were processed as described elsewhere (1). Kinetic constants were determined by GraphPad Prism software fitting the data directly to the Michaelis-Menten equation using nonlinear regression.

Results

The peptide derived from ALK reference sequence, containing Tyr-1278 was a good substrate of rALK, whereas peptides containing either Tyr-1282 or 15 Tyr-1283 were slightly affected by the enzyme (see Fig. 2). The Tyr-1278-derivative was phosphorylated with an efficiency even higher than that displayed by the peptide bearing three Tyr residues (Table I). To confirm that Tyr-1278-derivative is an optimal peptide substrate for ALK catalytic domain, we compared its phosphorylation degree with that displayed by polyGlu/Tyr (1:4), a 20 random polymer that is a very good substrate for most tyrosine kinases. Table II shows that Tyr-1278-derivative is a better ALK substrate than polyGlu/Tyr.

TABLE I

Kinetic constants for rALK with synthetic peptides.

25	PEPTIDE	V_{max} (pmol/min)	K_m (μ M)	Efficiency (V_{max}/K_m)
	ARDIYRASYYRKGGCAMLpvK	99.5	90.5	1.1
30	ARDIYRASFFRKGGCAMLpvK	186.3	109.4	1.7

TABLE II

Phosphorylation rates of model substrates by ALK catalytic domain.

Poly(Glu/Tyr) and peptide concentrations were 0.1 mg/ml and 400 μ M, respectively. Enzyme concentration was 10 units. Reported values represent 5 the means for three separate experiments. S.E.M. values were always less than 14%.

	SUBSTRATE	Phosphorylation degree (pmol/min)
	Poly(Glu/Tyr)	10.0
10	ARDIYRASFFRKGGCAMLVK	30.3

ELISA-based *in vitro* kinase assay

A Nunc Immuno 96 well plate was incubated overnight at 37°C with coating solution (125 μ l/ well) containing ALK peptide substrate (SEQ ID N. 1 or SEQ ID N. 2) at various concentrations in PBS. The wells were then washed 15 with 200 μ l of wash buffer (PBS-Tween 0.05 %) and left to dry for at least 2 hours at 37°C. The kinase reaction was performed by incubating kinase buffer (25 mM Hepes pH 7.5, 5 mM MnCl₂, 5 mM MgCl₂), 0.3 mM ATP and purified rALK at various concentrations in a total volume of 100 μ l/well at 30°C for 15 minutes. The reaction mix was then removed and wells were 20 washed 5 times with 200 μ l of wash buffer. Phosphorylated peptide was detected using 100 μ l/well of a mouse monoclonal anti-phosphotyrosine antibody (clone 4G10 UpstateBiotech Ltd) diluted 1:500 in PBS + 4% BSA. After 30 minutes incubation at room temperature the antibody was removed 25 and wells were washed as described above. 100 μ l of a secondary antibody (anti-mouse IgG, Horseradish Peroxidase linked whole antibody, from sheep, Amersham Pharmacia Biotech) diluted 1:1000 in PBS + 4% BSA was added to each well and the plate was incubated again for 30 minutes at room temperature before washing as above. The plate was developed using 100

μl/well TMB Substrate Solution (Endogen) and the reaction was stopped by adding an equal volume of H₂SO₄ 0.18 M. Finally, the absorbance was read at 450 or 490 nm using an Ultrospec® 300 spectrophotometer (Amersham-Pharmacia Biotech).

5 *Results*

We have shown that the ELISA-based kinase assay can be used to detect phosphorylated ALK peptide substrates (SEQ ID N.1 and N.2) and hence the activity of purified rALK. In initial experiments we performed the assay in the presence and absence of both purified rALK and the peptide substrate SEQ ID N.2, in order to determine if a specific increase in absorbance at 450 nm (A450) could be observed (Figure 3A). Indeed in the presence of both rALK and substrate a dramatic increase in A450 was observed. In contrast, in the absence of either peptide or rALK or both, A450 was low, indicating a low level of background absorbance. To determine the kinetics of substrate phosphorylation we measured A450 after various incubation times (Figure 3B). For both peptides the kinetics of the reaction was similar with maximum phosphorylation achieved after 10 minutes.

To compare the efficiency of the two peptides in the ELISA kinase assay and to determine the optimal concentration of peptide to use we performed standard curves for both peptides. Results show that increasing the amount of peptide substrate increased A450 and hence rALK kinase activity. For the peptide substrate SEQ ID N.2, maximum A450 and hence phosphorylation was observed at approximately 200 μM, compared with only 25 μM for peptide substrate SEQ ID N.1 (Figures 4A and B). This indicates that peptide substrate SEQ ID N.1 is more efficient as a substrate for rALK.

To observe inhibition of kinase activity it is essential that we use an appropriate concentration of enzyme, i.e. in the linear range of a standard

curve. An ELISA was performed using 206 μ M SEQ ID N.2 or 42 μ M SEQ ID N.1 and rALK ranging from 0 – 220 ng. The linear range of the curve for both substrates was approximately between 0 – 15 ng of rALK, after which the curves reached plateau (Figure 5).

5 The effect of the inhibitor staurosporine on rALK activity was assessed using 42 μ M peptide substrate SEQ ID N.2. A solvent control containing DMSO at corresponding concentrations was also performed. Staurosporine markedly inhibited rALK activity reaching maximum inhibition at 1 μ M and with an estimated $IC_{50} = 300$ nM (Figure 6). The solvent (DMSO) alone had
10 no substantial effect on rALK activity.

REFERENCES

[1] Glass, D.B., Masaracchia, R.A., Feramisco, J.R. and Kemp, D.E. (1978) Anal. Biochem. 87, 566-575.

5 [2] Till, J.H., Ablooglu, A.J., Frankel, M., Bishop, S.M., Kohanski, R.A. and Hubbard S.R. (2001) J. Biol. Chem. 276, 10049-10055.

CLAIMS

1. A method for detecting ALK tyrosine kinase activity, which comprises the following steps:

5 i) incubating the ALK protein or a functional derivative thereof with a peptide substrate selected from SEQ ID N. 1 or 2 in conditions suitable for phosphorylation of the peptide;

 ii) detecting the phosphorylated peptide.

2. A method according to claim 1, wherein the peptide has sequence SEQ ID
10 N. 1.

3. A method according to claim 1, wherein purified ALK protein or an ALK-containing preparation is used.

4. A method according to claim 3, wherein said preparation is a cell lysate.

5. A method according to claim 1, wherein said functional derivative
15 contains the entire catalytic domain of ALK spanning residues 1116-1392 of
ALK sequence,

6. A method according to claim 5, wherein said functional derivative is a fragment of ALK protein extending from residue Leu¹⁰⁷³ to Ala¹⁴⁵⁹.

7. A method according to claim 6, which comprises the steps of:

20 a) adhering a peptide of SEQ ID N. 1 or 2 to a solid phase;

 b) incubating the solid phase with said ALK fragment in conditions suitable for tyrosine phosphorylation;

 c) washing the solid phase;

 d) incubating the solid phase with an anti-phosphotyrosine antibody (primary antibody) in conditions suitable for antigen-antibody binding;

25 e) washing the solid phase;

 f) incubating the solid phase with an enzyme-conjugated antibody

(secondary antibody) recognizing the primary antibody in conditions suitable for the binding of primary and secondary antibodies, so that a ternary immune complex is formed;

g) washing the solid phase;

5 h) measuring the enzymatic activity of the immune complex wherein the measured activity is proportional to the amount of tyrosine-phosphorylation.

8. A method according to claim 7, wherein the enzyme conjugated to the antibody is Horse-Radish peroxidase.

10 9. A method according to claim 7, wherein the enzymatic activity is detected by colorimetric reaction.

10. A method according to any previous claims, for the identification of compounds that modulate ALK tyrosine-kinase activity.

11. A method according to claim 10, which comprises the steps of

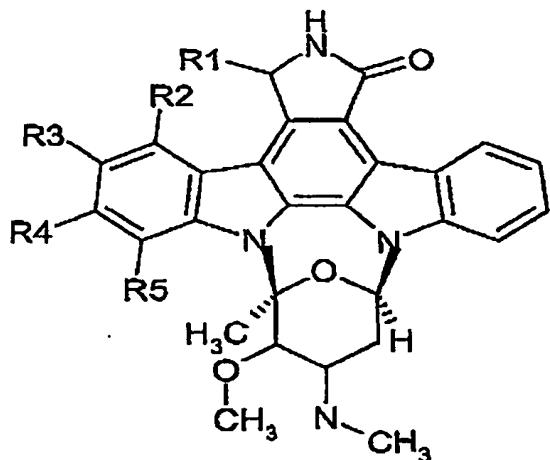
15 i) incubating ALK protein or a functional derivative thereof with a peptide selected from SEQ ID N. 1 or 2 in the presence of a candidate compound (a) in conditions suitable for peptide phosphorylation;

ii) detecting the phosphorylated peptide thus formed;

20 12. A method according to claims 10-11, wherein the ALK-modulating activity of the candidate compound is compared to that of a reference compound which is assayed under the same conditions as the candidate compound.

13. A method according to claim 12, wherein the reference compound is 25 staurosporine.

14. A method according to claim 12, wherein the reference compound is a staurosporine derivative of general formula (I):



Wherein R1 and R2, independently of one another, are selected from halogen, preferably chlorine, phenyl or C1-C3 alkyl optionally substituted with one or more halogens; R3 is hydroxyl; R4 is hydroxyl or hydroxymethyl; R5 is C1-C3 alkyl, optionally halo-substituted, or benzyl.

- 5 15. A peptide useful as ALK substrate selected from SEQ ID N. 1 or 2.
16. A peptide according to claim 15, which is SEQ ID N. 1.
17. The use of a peptide according to claim 15 or 16 for the determination of ALK tyrosine-kinase activity.
18. The use of a compound of formula (I), as per claim 14, for the preparation of a medicament for the treatment of ALK-related tumors, especially anaplastic large cell lymphomas and non-Hodgkin lymphomas.
- 10 19. A kit for detecting ALK tyrosine-kinase activity according to claims 1-14, which comprises a peptide of SEQ ID N: 1 or 2 and an anti-phosphotyrosine antibody.
- 15 20. A kit according to claim 19, containing an additional component selected from reagents for colorimetric reactions, buffers, diluents, detergents, stabilizers, staurosporine or a derivative thereof as per claim 14.

SEQUENCE LISTING

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<120> ANAPLASTIC LYMPHOMA KINASE ASSAY, REAGENTS AND COMPOSITIONS
THEREOF

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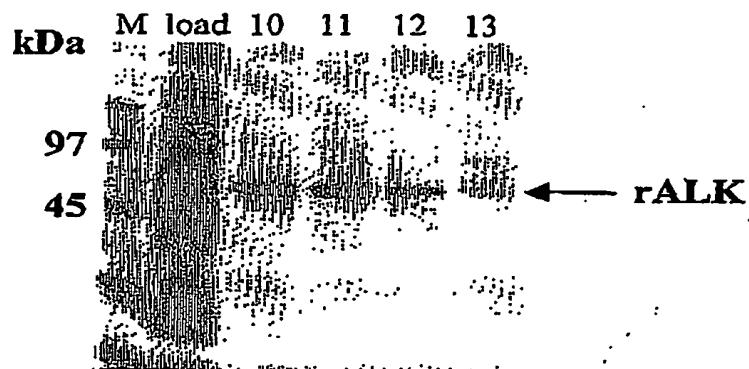
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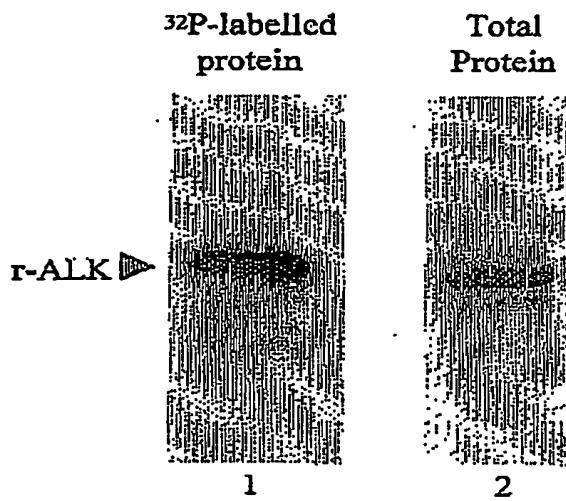
Sheet 1/6

Figure 1

A.

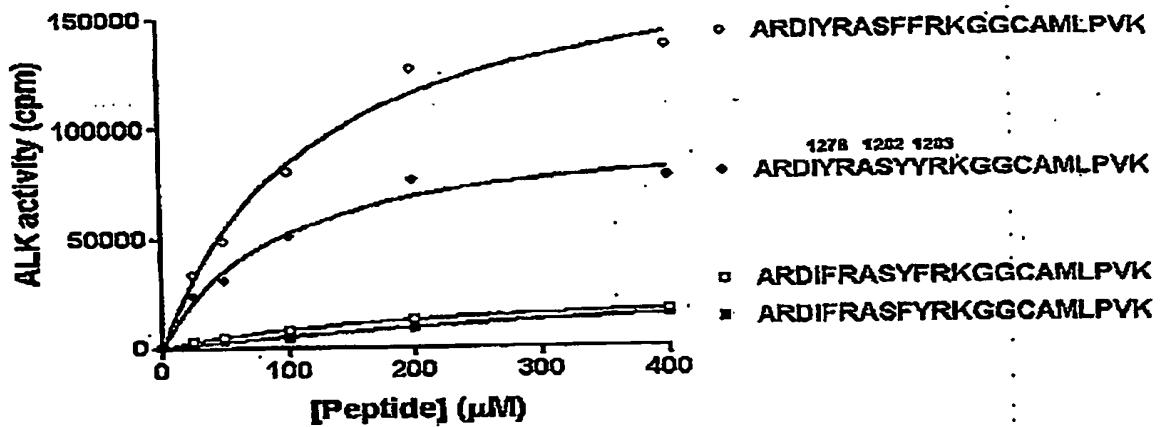


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Sheet 2/6

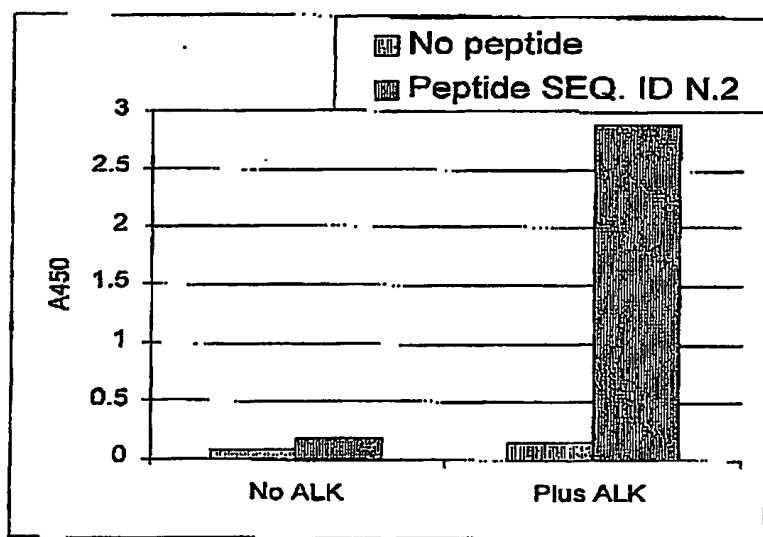
Figure 2



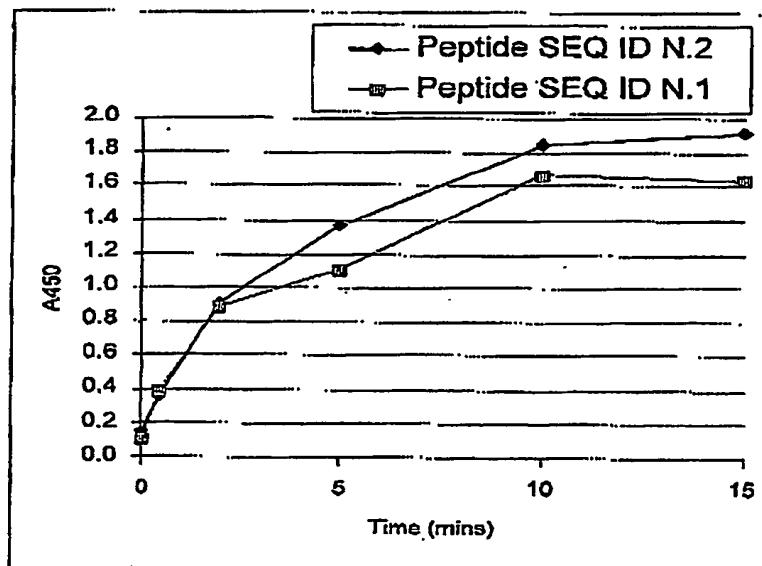
Sheet 3/6

Figure 3

A.

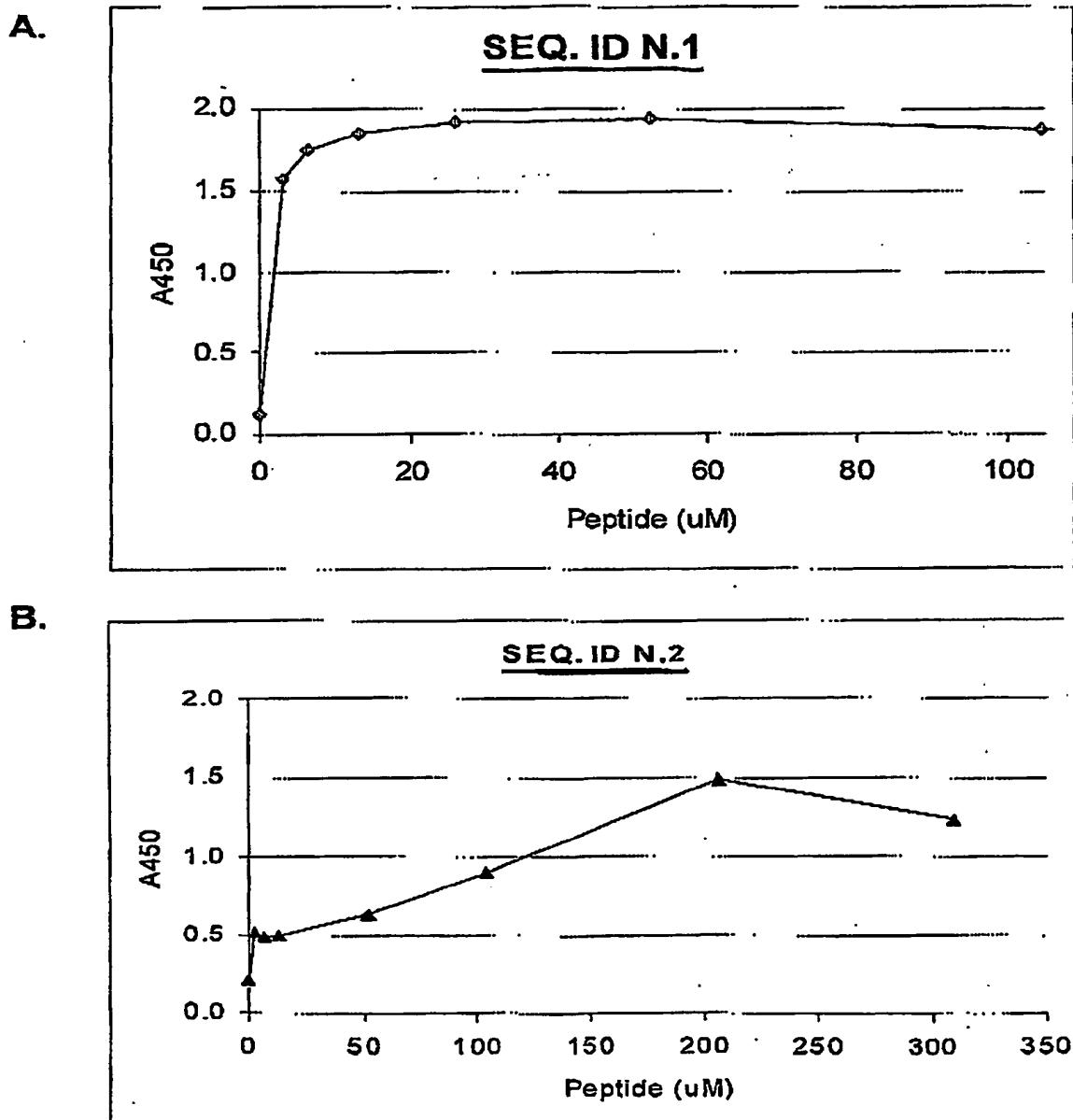


B.



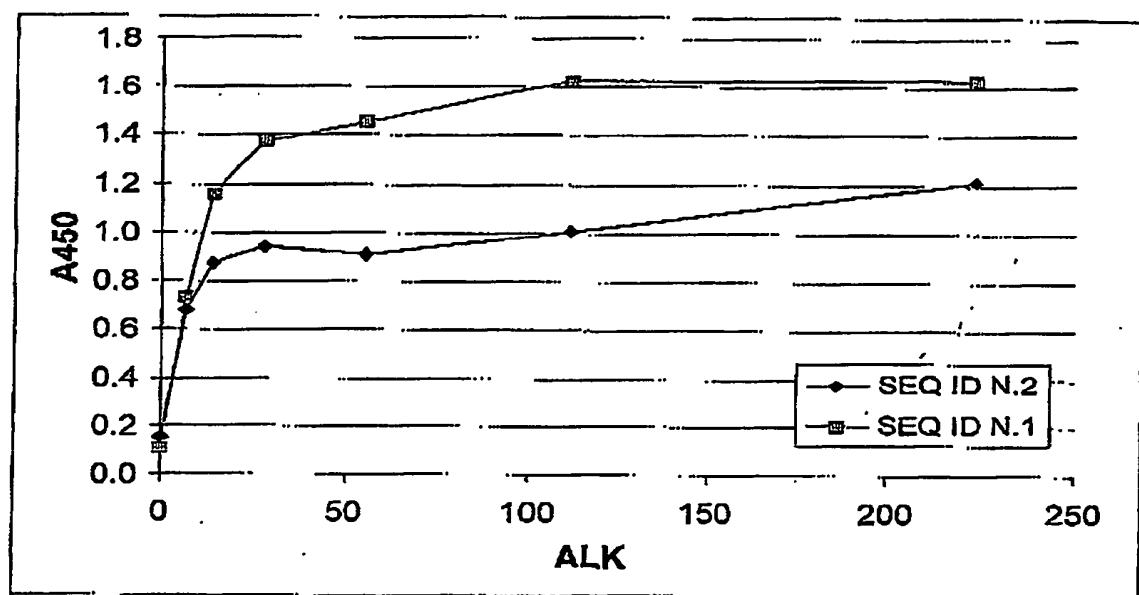
Sheet 4/6

Figure 4



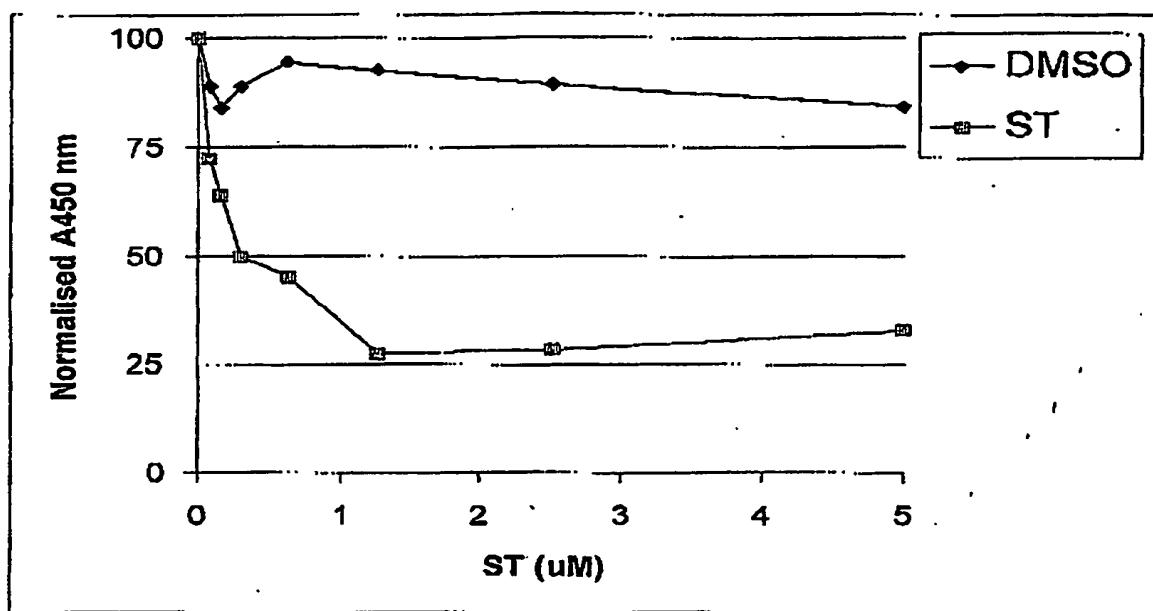
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Figure 5



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Figure 6



ABSTRACT**ANAPLASTIC LYMPHOMA KINASE ASSAY, REAGENTS AND COMPOSITIONS THEREOF**

5

Disclosed are methods for detecting ALK tyrosine kinase activity and for the identification of compounds that modulate ALK activity, peptide substrates, reagents and compositions thereof.

EP 04 2185



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